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In Vitro Fertilization and Embryo Development of Vitrified Ovine Oocytes Stressed in Sucrose

ITA DJUWITA[1*], ARIEF BOEDIONO[1], SRIHADI AGUNGPRIYONO[1], IMAN SUPRIATNA[2], MOZES TOELIHERE[2], YUHARA SUKRA[1]

[1]Department of Anatomy, [2]Department of Reproduction and Obstetric, Faculty of Veterinary Medicine, Bogor Agricultural University, Kampus Darmaga, Bogor 16680

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Experiments were conducted on the morphology, fertilization and embryo development rate of vitrified ovine oocytes matured *in vitro*. Three vitrification solutions were used for vitrification. PBS supplemented with 1% BSA, 30% ethylene glycol was added by one of three different sucrose concentrations, 1.00 M (VS_1), 0.50 M (VS_2), and 0.25 M (VS_3). The results showed that the percentages of normal vitrified oocytes after warming were 78 and 63% in VS_1 and VS_2, respectively, which was significantly higher as compared for VS_3. The fertilization rates were 59 and 66% in VS_1 and VS_2, respectively, which were also significantly higher as compared with VS_3 (35%). Zygote viability after 18 h was 57; 43; and 40%, for VS_1,VS_2, and VS_3, respectively, which was not significantly different. The incidence of polyspermic penetration increased with increasing sucrose concentration, i.e 23, 11, and 9% in VS_1, VS_2, and VS_3, respectively, as compared with unvitrified oocytes (4%). The cleavage rate of vitrified oocytes in VS_1 was 13.2% which was significantly lower ($p<0.05$) compared to those of unvitrified control oocytes (70.0%). Hence, a high sucrose concentration is beneficial for maintaining the oocyte structure during the processes of vitrification and thawing, which ultimately results in increased *in vitro* fertilization rates.

INTRODUCTION

One of the most critical factor in the development of *in vitro* embryo production in mammals is the availability of viable and developmentally competent oocytes. A limiting factor in this context is the relative short fertile life of the mammalian oocyte. This limitation, could be overcome through cryopreservation (Park & Ruffing 1992). Female gametes (or germ lines) of laboratory and domestics animals could be preserved indefinitely or banked for use in research and commercial application. Those applications were ranging from providing hamster ova for sperm penetration assays to research and development related to cloning and genetic engineering (Niemann 1991). The germplasm of valuable females, including genetically engineered individuals and endangered species, could be salvaged after loss of normal fertility or even death. However, it is generally recognized that the developmental competence of cryopreserved mammalian oocytes is extremely limited (Niemann 1991).

Matured oocytes that have been cryopreserved using an ultra-rapid method showed very low *in vitro* fertilization and embryo developmental rates (Park & Ruffing 1992; Rayos *et al*. 1994). This is presumably due to the ice cristal formation that causes membrane and cell organel damage (Aman & Parks 1994; Fukui *et al*. 1995; Vajta 1997).

Another method of cryopreservation is vitrification (Rall & Fahy 1985; Nakagata 1989; Arav *et al*. 1993) based on the solidification of a liquid by an extreme increase in viscosity during very rapid cooling. This solid, called glass (vitreus), has the molecular and ionic distribution of the liquid state, and, thus, avoids the potentially detrimental effects of extracellular and intracellular crystallization. However, vitrification requires the presence of a high concentration of cryoprotectant that could be damaging to cells by the osmotic stress or chemical toxicity. To minimize these undesired effects, multilevel concentrations of non-permeable cryoprotectants or extra-cellular cryoprotectants such as sucrose can be applied. Addition of sucrose into the embryo equilibration medium before cryopreservation resulted in an increase in embryo viability after thawing (Szell & Shelton 1986; Takahashi & Kanagawa 1990). Therefore, the purpose of this research was to examine the effect of sucrose addition in various concentrations i.e. 1.00, 0.50, and 0.25 M on the morphology, viability, *in vitro* fertilization and embryo development rates of *in vitro* matured oocytes.

MATERIALS AND METHODS

Oocyte Collection and *In Vitro* Maturation. Sheep ovaries were collected at a local abbatoir immediately after slaughter and transported to the laboratory in 30 to 35 °C isotonic saline containing penicillin-G (1000 U/ml) and streptomycin sulfate (0.2 µg/ml) within 2 h. Oocytes were aspirated from 2- to 4-mm follicles with a 20 G needle attached to a 5 ml syringe containing phosphate buffer saline (PBS, Gibco, USA) supplemented with 0.3% (v/v) bovine serum albumine (BSA, Sigma, USA) and 50 µg/ml gentamycin sulfate (Sigma, USA). Only oocytes enclosed in a compact multilayered cumulus invertment and with evenly granulated ooplasm were used. The oocytes were washed three times with a maturation medium (TCM-199, Earle's salt with sodium bicarbonate and L-glutamine)

*Penulis untuk korespondensi, Tel. +62-251-421823 Fax. +62-251-629464, E-mail: djuwitawiryadi@yahoo.com

supplemented with 10% (v/v) inactivated new born-calf serum (NCS, Sigma, USA); 10 µg/ml follicle stimulating hormone (FSH, Denka Pharmaceutical Japan) and gentamycin sulfate 50 µg/ml, and the treated oocytes were then cultured in 5% CO_2 at 38.5 °C for 22 h.

Vitrification of *In Vitro* Matured Oocytes. After maturation culture, oocytes with cumulus cells were washed in PBS and were then cryopreserved by vitrification according to the method of Tada *et al.* (1994), with slight modifications. The three groups of vitrification solution used were PBS supplemented with 1% BSA, 30% ethylene glycol and three different sucrose concentrations, i.e 1.00 M (VS_1), 0.50 M (VS_2), and 0.25 M (VS_3). The three groups of oocytes were tested with respect to the vitrification solution and pre-vitrification equilibration. For group I, oocytes were equilibrated in 400 ml, with of two concentrations of sucrose in PBS (0.25 M and then 0.50 M for 5 min each) at room temperature in a 30 mm suspension culture dish and then exposed for 30 sec at room temperature to 500 ml vitrification solution (VS_1) and loaded into a 0.25 ml PVC straws, each containing 10 oocytes. After 10 sec evaporation on liquid nitrogen vapor, the straws were plunged into liquid nitrogen and stored for a period ranging from 2 to 4 weeks. For group II, oocytes were equilibrated with 400 ml of the 0.25 M sucrose in PBS for 5 min at room temperature and vitrified in VS_2 solution as described for group I; while for group III, oocytes were directly exposed to and vitrified in the VS_3 solution as described for group I.

Warming. Vitrified oocytes were warmed in air for 10 sec and plunged into a 35 °C water bath. The content of the straws were emptied into a culture dish containing 0.50 M sucrose in PBS. After 5 min, the oocytes were washed 3 times using PBS and evaluated for their morphology (degenerated or normal). Only normal oocytes were fertilized *in vitro* using fresh semen.

***In Vitro* Fertilization.** *In vitro* fertilization was done in CR1aa (C.Rozenkrans 1 amino acid) medium containing 0.2 mM caffein and 5 mg/ml heparin. Fresh semen of a local ram was washed in 8-ml CR1aa medium containing 0.2 mM caffein and 0.3% BSA. The sperm pellet was resuspended and diluted in CR1aa medium containing 0.2 mM caffein, 2.0% BSA and 0.2 mM heparin to a final concentration of 10 x 10^6 sperm/ml. Groups of 10-20 vitrified oocytes were put into 100 ml droplets of sperm suspension and incubated in 5% CO_2 incubator at 38.5 °C for 18 h. Unvitrified oocytes were presenced for *in vitro* fertilization as a control.

***In Vitro* Development of Vitrified-Warmed Oocytes.** Eight hours after insemination, oocytes were washed 3 times and transferred to the culture medium (CR1aa) supplemented with 10% (v/v) NCS, 5 mg/ml insulin and 50 mg/ml gentamycin for development. Embryos incubation was carried ouf in 5% CO_2 incubator at 38.5 °C.

Evaluation and Examination. The morphology of the thawed oocytes was evaluated by light microscope. The oocytes were classified as degenerated (including oolemma disintegration, zona pellucida fracture, and inhomogenous ooplasma or normal). Evaluation of fertilization was assessed by visualizing pronucleus formation by the staining method of Iwasaki *et al.* (1990). Fertilized oocytes were washed using PBS and put into 100 ml of PBS containing bizbenzimidize (staining DNA in both live and dead cells) and propidium iodine (staining DNA in only dead cells), each with concentration of 1 mg/ml for 30 min. Stained oocytes were placed onto a slide and covered with a cover slip. Examination was done under the fluorescence microscope. Blue and pink colors in the pronuclei of the zygotes indicated live and dead cells, respectively (Iwasaki *et al*. 1990).

Experiment Design and Analysis. Experiments were repeated three times and data collected were analysed statistically using Analysis of Varian (ANOVA).

RESULTS

The Morphology of Oocytes Post Vitrification and Warming. Figure 1a showed dehydration of oocytes in the vitrification solution. The oocytes which had normal round cytoplasm after warming were defined as morphologically normal. The percentages of morphologically normal oocytes (Figure 1b) after vitrification and warming in VS_1 (1.00 M sucrose) and VS_2 (0.50 M sucrose) were 78 and 63%, respectively, which were significantly higher ($P<0.05$) than those in VS_3 (0.25 M sucrose) (40%), in Table 1. Vitrification and warming processes of oocytes also caused in some changes in the structures of the zona and/or vittelus as indicated by zona pellucida fracture (Figure 1c) and/or oolemma disintegration (membrane lysis) which lead to oocytes degeneration (Figure 1d). The percentages of degenerated oocytes after vitrification and warming in VS_1 (1.00 M sucrose) and VS_2 (0.50 M sucrose) were 22 and 37%, respectively, which were significantly lower ($P<0.05$) than those in VS_3 (0.25 M sucrose) (62%), in Table 1.

The *In Vitro* Fertilization and Embryo Cleavage Rates After Vitrification and Warming of the Oocytes. The *in vitro* fertilization rate of the normal oocytes after vitrification and warming in VS_1 and VS_2 were 82 and 77%, respectively, which were significantly higher ($P<0.05$) than those in VS_3 (44%), but not significantly different ($p>0.05$) from unvitrified control oocytes (84%), in Table 2. However, high concentrations of sucrose in the solution increased the incidence of polyspermic penetration, as shown by high percentage of polyspermic penetration (>2PN) i.e. (23, 11, and 9%) in VS_1, VS_2, and VS_3, respectively, as compared with unvitrified control oocytes (4%). The percentages of unfertilized oocytes in VS_1, VS_2, and unvitrified control were 16, 18, and 34% significantly lower than in VS_3 (56%). The zygote viability at 18 h *in vitro* insemination were not significantly different ($P>0.05$) between the three treatments, i.e. 57, 43, and 40%, for VS_1, VS_2, and VS_3, respectively, but were significantly lower ($p<0.05$) than unvitrified control oocytes (74%) (Table 2).

The embryo cleavage rates of vitrified oocytes in VS_1 were 59.0 and 13.2% were significantly lower ($p<0.05$) than those of exposed oocytes (66.7 and 60.0%) and unvitrified control oocytes (77.0 and 70.0%), as shown in Table 3.

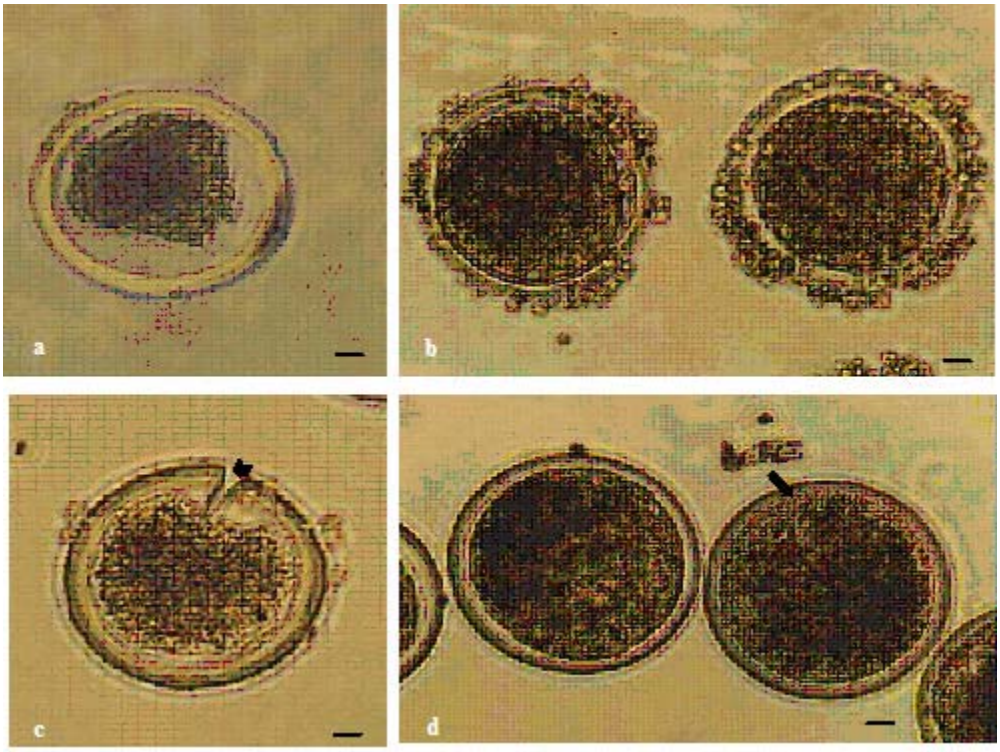

Figure 1. Ovine oocytes, pre- (a) and post-vitrification and warming (b-d). (a) Oocytes in vitrification solution; (b) Oocytes with normal morphology; (c) Oocytes with zona fracture indicated by arrow head; (d) Degenerated oocytes with lysis membrane indicated by arrow. Bar = 20 μm.

Table 1. Oocyte morphology after vitrification in media with various sucrose concentration from 3 replicates

Sucrose concentration (M)	No. of oocytes	Oocytes morphology (%)			
		Abnormal			Normal
		Zona frakture	Degenerated	Total	
1.00 (VS_1)	63	7 (11)a	7 (11)a	14 (22)a	49 (78)a
0.50 (VS_2)	77	16 (21)a	12 (15)a	28 (36)a	49 (64)a
0.25 (VS_3)	107	38 (36)b	26 (24)b	64 (60)b	43 (40)b

Values within column with different superscripts are significantly different (P<0.05)

Table 2. Viability and fertilization rate after vitrification in media with various sucrose concentration from 4 replicates

Sucrose concentration (M)	No. of oocytes	Pronucleus formation (%)				Oocytes viability** (%)
		2 PN	>2 PN	Fertilized*	Unfertilized	
Control (Unvitrified)	56	45 (77)a	2 (4)b	47 (84)a	9 (16)a	39 (74)b
1.00 (VS_1)	44	26 (59)a	10 (23)a	36 (82)a	8 (18)a	25 (57)a
0.50 (VS_2)	35	23 (66)a	4 (11)b	27 (77)a	8 (23)a	15 (43)a
0.25 (VS_3)	43	15 (35)b	4 (9)b	19 (44)b	24 (56)b	17 (40)a

*Fertilized counted as 2 PN (pronuclei) plus >2 PN, **Oocytes viability at 18 h after *in vitro* insemination. Values within column with different superscripts are significantly different (P<0.05)

Table 3. Cleavage rates of exposed and vitrified *in vitro* matured oocytes

Oocytes treatment	No. of oocytes cultured	Cleavage rates (2-8 cell) (%)
Control (Unvitrified)	66	46(70.0)a
Exposed oocytes to 1.00 M (VS_1)	60	36(60.0)a
Vitrified oocytes to 1.00 M (VS_1)	68	9 (13.2)b

Values within column with different superscripts are significantly different (P<0.05)

DISCUSSION

It was demonstrated that, vitrification and warming causes damage of the oolemma, zona pellucida and ooplasma which lead to oocytes degeneration. Zona pellucida fractures (ruptures) and lysis of oolemma was the most obvious morphological damages in frozen-thawed oocytes reported by Kubota *et al.* (1998). However, in this research the occurrence of this phenomenon was reduced by increasing

the sucrose concentration in the vitrification solution. Pre-vitrification equilibration with a multilevel sucrose solution in this study not only could facilitated the dehydration process but also could reduced of the tocixity effect of the 30% ethylene glycol. This results is in line with the previous report by Tada *et al.* (1994) that pre-freezing equilibration with a sucrose solution is known to improve the cryosurvival of mouse oocytes. However, after vitrification and thawing, there was great differences in the percentage of normal oocyte recovery between those vitrified in solution containing 1.00 M and 0.50 M with those vitrified in solution containing 0.25 M sucrose. This is in contrast with the present study, which may be due to species differences. The membrane permeability of the cell increases as the volume of the is smaller (Mazur *et al.* 1976).

The presence of high concentrations of cryoprotectant (30% ethylene glycol) in the vitrification solution decreases the probability of intracellular crystallization which is considered to cause damage during rapid cooling (Arav *et al.* 1993), but can also cause toxic and osmotic injuries to the oocytes even without cooling. Sucrose as a non-permeating cryoprotectant, that tend to reduce these toxicity effects (Shuwen *et al.* 1998); while equilibration with multilevel concentrations of sucrose can minimize the osmotic injury as shown from the percentages of degenerated oocytes in the three groups (Tada *et al.* 1994). Sucrose as macro molecule (non-permeating) increased the water dehydration from the cytoplasm a step which is critical in vitrification process (Arav *et al.* 1993). The shrinkage of the oocyte and consequently the amount of water inside the oocytes that may crystallize during rapid cooling and warming is lower (Rall 1987).

The *in vitro* fertilization rates of the normal oocytes after vitrification and warming were not significantly different ($p>0.05$) in VS_1 and VS_2 from those observed in unvitrified control oocytes. However, high concentrations of sucrose in the solution increased the incidence of polyspermic penetration. Similar result have been reported by Rayos *et al.* (1994). Some structures such as the oolemma, zona pellucida and cortical granules which play roles in establishing the block again polyspermic penetration may have been altered during the vitrification process (Arav *et al.* 1993; Hyttel *et al.* 2000). Metaphase II spindles (cytoskeleton) was also been reported to be sensitive to temperature cooling between 25 ^oC and 4 ^oC (Aman & Park 1994; Park & Ruffing 1992). The viability of the zygote at 18 h after *in vitro* insemination were significantly different in VS_1 and VS_2, as compared with unvitrified control. This indicate that, although the oocytes were of normal morphology after the processes of vitrification and warming, some may have suffered intracellular damages which were not evident under the light microscope. This results were supported also by the embryo cleavage rate of vitrified oocytes (13.2%) which were significantly different from those of unvitrified control oocytes (70.0%).

REFERENCES

Aman RR, Park JE. 1994. Effect of cooling and rewarming on the meiotic spindle and chromosome of *in vitro* matured bovine oocytes. *Biol Reprod* 50:103-110.

Arav A, Shehu D, Mattioli M. 1993. Osmotic and cytotoxic study of vitrification of immature bovine oocytes. *J Reprod Fert* 99:353-358.

Fukui E, Xia L, Downey BR. 1995. Ultrastructural changes in bovine oocytes cryopreservation by vitrification. *Cryobiology* 32:139-156.

Hyttel P, Vajta G, Callasen H. 2000. Vitrification of bovine oocytes with the open pulled straw method: ultra-structural consequences. *Mol Reprod Dev* 56:80-88.

Iwasaki S, Yoshiba N, Ushijima H, Watanabe S, Nakahara T. 1990. Morphology and proportion of inner mass of bovine blastocysts fertilized *in vitro* and *in vivo*. *J Reprod Fert* 90:279-284.

Kubota C *et al.* 1998. *In vitro* and *in vivo* survivals of frozen-thawed bovine oocytes after IVF, nuclear transfer and parthenogenetic activation. *Mol Reprod Dev* 51:281-286.

Mazur P, Rigopoulos M, Zhu SE, Sakurai T, Machida T. 1976. Preliminary estimates of the permeability of mouse ova and early embryos to glycerol. *Biophys J* 16:232a (Abstract).

Nakagata N. 1989. High survival rate of unfertilized mouse oocytes after vitrification. *J Reprod Fert* 87:478-483.

Niemann H. 1991. Cryopreservation of ova and embryos from livestock: current status and research needs. *Theriogenology* 35:109-124.

Park JE, Ruffing NA. 1992. Factors affecting low temperature survival of mammalia oocytes. *Theriogenology* 1:59-73.

Rall WF. 1987. Factors affecting the survival of mouse embryos cryopreserved by vitrification. *Cryobiology* 24:387-402.

Rall WF, Fahy GM. 1985. Ice free cryopreservation of mouse embryos at -196 ^oC by vitrification. *Nature* 313:573-575.

Rayos AA, Takahashi Y, Hishinuma M, Kanagawa H. 1994. Quick freezing of unfertilized oocytes using ethylene glycol with sucrose or trehalose. *J Reprod Fert* 100:123-129.

Shuwen Z, Yoshizawa M, Muramatsu S. 1998. Analysis of fertilizability of bovine oocytes cryopreserved in various cryoprotectants after *in vitro* maturation and their chromosomes at the first cleavage division. *J Mamm Ova Res* 15:37-42.

Szell A, Shelton JN. 1986. Role of equilibration before rapid freezing of mouse embryos. *J Reprod Fert* 78:699-703.

Tada N, Sato M, Kasai K, Ogawa S. 1994. Successful *in vitro* and *in vivo* development of cryopreserved mouse oocytes fertilized by cryopreserved mouse epididymal spermatozoa. *J Reprod Fert* 40:65-70.

Takahashi Y, Kanagawa H. 1990. Effect of equilibration on the viability of frozen -thawed mouse embryos after rapid freezing. *Mol Reprod Dev* 26:105-110.

Vajta G. 1997. Vitrification of bovine oocytes and embryos *Embryo Transfer Newslet* 15:12-18.